Synthesis and Biological Evaluation of Novel Potent Antagonists of the Bombesin/Gastrin Releasing Peptide Receptor[1]

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This paper reports the synthesis and antagonist activity of 20 C-terminal analogues of gastrin releasing peptide (GRP). The ability of each analogue to inhibit bombesin (BN) stimulated amylase release from rat pancreatic acini was determined, and those showing antagonist activity were further evaluated for their ability to inhibit BN-stimulated [^{3}H]thymidine uptake in serum-starved 3T3 cells. The assays also included two known peptide antagonists, **C** (Leu^{14},$\psi^{13,14}$]BN) and **H** (*N*-pivaloyl-GRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide) as positive controls. On the basis of these assays we suggest that a des-Met^{27},Leu^{26}-ψ[$CH_2NHCOCH_3$]GRP C-terminal octapeptide imparts antagonist activity. The two most active compounds are peptides **14** ([D-Phe^{19},Leu^{26}-$\psi(CH_2NHCOCH_3)$]GRP$_{19-26}$) and **18** ([D-Phe^{19},Gln^{20},Leu^{26}-$\psi(CH_2NHCOCH_3)$]GRP$_{19-26}$). In their ability to inhibit BN-stimulated [^{3}H]thymidine uptake, the IC$_{50}$ of peptides **C**, **H**, **14**, and **18** were 43.2, 31.2, 2.7, and 32.5 nM, respectively. In conclusion, the novel C-terminal ψ[$CH_2NHCOCH_3$] bond promises to be a useful peptide backbone modification for imparting antagonism in GRP/BN analogues.

Introduction

Small cell lung cancer (SCLC) is one of the more common fatal malignancies and worldwide its incidence is increasing.[2] SCLC comprises about 20–25% of all lung cancers and only 5% of patients survive 2 years after diagnosis. Most SCLC lines produce gastrin releasing peptide (GRP), a mammalian 27-amino acid peptide which has very similar biological actions to that of bombesin (BN).[3] BN is a 14-amino acid peptide which was originally isolated from amphibian skin. SCLC cell lines express high-affinity receptors for GRP and exhibit a mitogenic response to this peptide, thus suggesting that GRP can function as an autocrine growth factor for human SCLC.[4] On the basis of this, the interruption of the supposed autocrine growth loop with GRP or BN antagonists should suppress the growth of this tumor. As a result, there has been considerable interest in the design and development of competitive BN or GRP receptor antagonists as possible therapeutic agents.[5-8] Furthermore, GRP appears to be important in other disease states and thus GRP antagonists may have additional clinical utility.[7,9]

However, there is still some doubt as to how useful BN/GRP antagonists would be in the treatment of SCLC. Whereas Heimbrook et al. reported[10] the development of potent GRP antagonists, they were unable to demonstrate their effectiveness at inhibiting the growth of SCLC cells in vitro. Contrary to this, Mahmoud et al.[11] were able to demonstrate that a BN analogue inhibited "SCLC growth using a clonogenic assay in vitro and xenograft formation in nude mice in vivo".

The mitogenicity has been shown to be due to the C-terminal segment, GRP$_{14-27}$; GRP$_{1-16}$ did not stimulate

	18										25
GRP$_{14-27}$	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2

	5									10					
BN	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2

SCLC growth.[12] Both BN and GRP$_{14-27}$ are known to stimulate the clonal growth of SCLC cells in vitro.[12] It has also been demonstrated that the C-terminal eight

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Table I. Peptides Synthesized

no.	structure
1	[D-Phe^{21},D-Trp^{23,25},Leu^{27}]GRP$_{18-27}$
2	[D-Arg^{17},D-Phe^{21},D-Trp^{23,25},Leu^{27}]GRP$_{17-27}$
3	[D-Phe^{19},Leu^{26}-ψ(CH$_2$NCHO)]GRP$_{18-27}$
4	[D-Phe^{19},Leu^{26}-ψ(CH$_2$NCHO)]GRP$_{19-27}$
5	[Leu^{26}-ψ(CH$_2$NCHO)]GRP$_{18-27}$
6	[N-Me-Phe^{19},Leu^{26}-ψ(CH$_2$NCHO)]GRP$_{19-27}$
7	[N-Me-D-Phe^{19},Leu^{26}-ψ(CH$_2$NCHO)]GRP$_{19-27}$
8	[Gly^{19},Asn^{20},$\psi^{26,27}$]GRP$_{19-27}$
9	[$\psi^{26,27}$]GRP$_{21-27}$
10	[D-Phe^{20},$\psi^{26,27}$]GRP$_{20-27}$
11	[Ac-Trp^{21},$\psi^{26,27}$]GRP$_{21-27}$
12	[N-Me-Phe^{20},$\psi^{26,27}$]GRP$_{20-27}$
13	[Ac-Gln^{19},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
14	[D-Phe^{19},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
15	[Gln^{19},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
16	[Ac-D-Phe^{19},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
17	[Ac-D-Phe^{19},Gln^{20},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
18	[D-Phe^{19},Gln^{20},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
19	[Ac-Gln^{19},Trp(CHO)^{21},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$
20	[D-Phe^{19},Trp(CHO)^{21},Leu^{26}-ψ(CH$_2$NHCOCH$_3$)]GRP$_{19-26}$

amino acids of GRP possesses full receptor-binding activity and can elicit a full biological response.[13] Since GRP$_{18-27}$ (neuromedin C, NMC) and BN$_{5-14}$ are identical except for one amino acid (His^{20} of GRP vs Gln^{7} of BN), many of the GRP/BN antagonist studies have used both of these partial structures in attempts to design potent antagonists.[14]

This paper describes our efforts in the preparation of 20 new peptides (Table I) containing modifications of the C-terminal portion of GRP, thus resulting in several novel peptides which are potent and specific antagonists of the GRP/BN receptor.

Peptides 1 and 2 were modeled after [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}] substance P, an analogue prepared by Woll and Rozengurt,[13] which was reported to be a potent BN antagonist. In order to avoid the side reaction of the oxidation of Met to Met(0), they replaced this residue with Leu. Substance P (SP), which has only slight amino acid homology with GRP$_{17-27}$, does not itself inhibit GRP binding nor enhance [^{3}H]thymidine incorporation in Swiss 3T3 cells. However, in Swiss 3T3 cells, the SP analogue did inhibit mitogenesis that had been stimulated by GRP.[15]

In BN, Coy et al.[16] has reported that substituting Leu^{14} for the easily oxidized Met^{14} afforded a peptide which had about a third of the biological potency and receptor binding affinity as the parent. When we began this study, des-Met analogues of the C-terminus of GRP and BN had not yet been reported.[5,13,17,18] It is also known that replacement of selected peptide bonds by the pseudo(CH$_2$NH) (ψ-bond) group is one of the possible peptide backbone modifications that affords receptor antagonists.[16,19] Therefore, we planned to incorporate $\psi^{26,27}$(CH$_2$NH) into GRP peptides **3–12**. We chose this position because it has been shown[20] that [$\psi^{13,14}$(CH$_2$NH),Leu^{14}]BN is a strong BN receptor antagonist and it was thought that retention of the natural Met residue in GRP might afford greater receptor binding and more potent antagonists.

Jensen and Coy[14] have reported that four classes of BN receptor antagonists have been identified. Since two of these, the [des-Met^{14}]BN and [ψ(CH$_2$NH)]BN analogues, have proven to be potent antagonists, we decided to investigate a new class that would incorporate both of these changes into a C-terminal portion of GRP. Therefore, we prepared a series of [des-Met^{27}]GRP analogues containing a C-terminal ψ(CH$_2$NHCOCH$_3$) group: peptides **13–20**. These peptides were modeled after the potent des-Met antagonists, [D-Phe^{6}]BN$_{6-13}$ alkylamides[14] and Ac-GRP$_{20-25}$ alkylamides.[7] Furthermore, peptides **13–20**, in which the Leu carbonyl has been replaced with a methylene, would be expected to be antagonists if one considers the type II′ β-bend model proposed by Coy et al.[16] for agonist activity. In this model,[16] replacement of the Leu carbonyl by methylene destroys a key hydrogen bond, resulting in a conformational shift which causes loss of biological (agonist) activity, but not the ability to bind to the receptor and act as an antagonist. It is interesting to note that the Ac-GRP$_{20-25}$ alkylamides[7] described above, like peptides **13–20**, also contain a Leu in which the carbonyl group has been replaced with a methylene unit and are potent antagonists. As will be discussed below, this new class of [des-Met^{27}]GRP analogues containing a C-terminal ψ(CH$_2$NHCOCH$_3$) group has afforded two potent and specific GRP/BN receptor antagonists.

Results and Discussion

Chemistry. All of the peptides were synthesized in a manual synthesizer using typical Merrifield solid-phase conditions[21] and either *p*-methylbenzhydrylamine (MBHA) or benzhydrylamine (BHA) resins. All amino acids were coupled as their N^{α}-Boc derivatives, and the trifunctional amino acids Arg(tos), His(Bom), and Trp(For) were further protected; the latter was sometimes used with side chain unprotected.

Peptides **1–12** were synthesized on MBHA resin because it affords C-terminal amides and is more susceptible to the final hydrogen fluoride (HF) cleavage conditions relative to the BHA resin.[21] On the other hand, peptides **13–20** were synthesized on BHA resin because in this series the peptide to resin linkage is more stable to repeated trifluoroacetic acid (TFA) treatment.

Each amino acid residue was added to the growing peptide using single or double coupling, the choice determined by the results of a quantitative ninhydrin assay.[22] All the residues except His(Bom), Gly, Gln, Asn, and Arg(Tos) were single coupled as their symmetrical anhydrides in dichloromethane (DCM). The second

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couplings, if needed, were performed as hydroxybenzotriazole (HOBt) esters in dimethylformamide (DMF). The other five Boc-amino acids were initially coupled as their HOBt esters in DMF/DCM (2/1), while the second coupling, if necessary, was performed in DMF as the HOBt ester.

Boc-Asn and Boc-Gln were coupled as their HOBt esters in order to minimize nitrile formation.[23] To avoid the possibility of a diacylamide side reaction occurring as the result of using Boc-Gly symmetrical anhydride, the somewhat less powerful acylating agent, Boc-Gly-OBt was used in the peptide syntheses.[24] Boc-Arg(Tos) was not used as its symmetrical anhydride because it can cause a double-insertion reaction[25] as well as formation of a lactam.[26]

The introduction of the $\psi(CH_2NH)$ peptide bond isostere into peptides **3–12** was accomplished on solid phase following the method of Sasaki and Coy.[27] Furthermore, Coy et al. have shown[19] that racemization during solid-phase reductive alkylation can be kept to zero by controlling the temperature and amount of $LiAlH_4$ used in the reduction conditions that we used. Boc-Gly-ONp was used in the peptide syntheses after the introduction of the $\psi(CH_2NH)$ bond in order to minimize acylation of the secondary amine by Boc-Gly.[27]

The presence of the formyl group on the secondary amine, $\psi(CH_2NCHO)$, in peptides **3–7** was unexpected. The original plan was for these five peptides to contain the $\psi(CH_2NH)$ function between Leu^{26} and Met^{27}. The TFA salt of Met bound to MBHA resin was used to introduce the $\psi(CH_2NH)$ bond by reaction with Boc-leucinal[28] followed by reduction with sodium cyanoborohydride in acidified dimethylformamide (DMF).[27] This same resin was used to elaborate all five peptides, **3–7**. After peptides **3–7** were isolated and the major component purified by semipreparative HPLC, analysis by fast atom bombardment mass spectrometry (FABMS) showed protonated molecular ions $(M + H)^+$ 28 atomic mass units (amu) higher than expected for each peptide, suggesting the presence of a formyl (CHO) group.

In order to confirm the position of the additional CHO group, we subjected peptides **3**, **5**, and **6** to collision induced decomposition mass spectrometry (CID-MS) in a tandem mass spectrometer system (MS/MS).[29,30] This methodology of MS/MS has been found to be a powerful tool in determining the amino acid sequence of peptides and in the structural elucidation of modified peptides. The

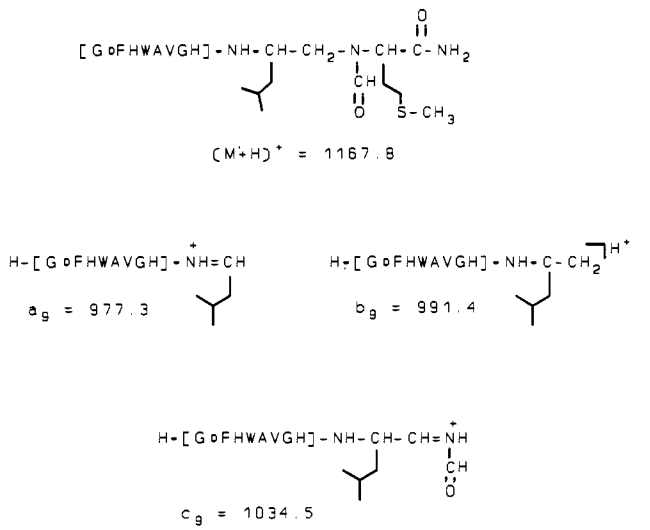

Figure 1. Ions a_9, b_9, and c_9 from the CID-MS fragmentation of peptide **3**.

fragment ion nomenclature used here has been summarized by Biemann.[30] The CID spectrum of peptide **3** showed an $(M + H)^+$ ion of m/z 1167.8 as well as an a_9 ion at 977.3 and a b_9 ion at 991.4, indicating no additional mass of 28 to the peptide through nine amino acids. Furthermore, the b_9 ion indicates that the $\psi(CH_2NH)$ exists between Leu^{26} and Met^{27}. The observation of the key ion, c_9 at 1034.5, clearly places the CHO on the Met^{27} secondary amine (see Figure 1). Similar ions were observed in the CID spectra of peptides **5** and **6**, and since peptides **4** and **7** were also derived from the same MBHA resin, this confirms the presence of the CHO group on the secondary amine, $\psi(CH_2NCHO)$, in peptides **3–7**.

Presently we are not able to explain how the incorporation of the CHO group occurred in peptides **3–7**. Although it is known that formaldehyde adducts can form during acidolytic hydrogen fluoride (HF) cleavage when the benzyloxymethyl (Bom) protecting group is used for His, the CID-MS results do not support any of the reported side products.[31] Furthermore, peptides **8–12** do not show an increase of 28 amu in their molecular ions even though His(Bom) was also used in their syntheses. One possible cause of the formylation might be due to a contaminant in the DMF solvent used.[32] However, the suspect DMF had been exhausted and we could not again secure this lot from the manufacturer in order to further study this possibility. Different lots of DMF from the same manufacturer or different manufacturers, which were used in the preparation of peptides **8–12**, never gave rise to similar formylated peptides.

The Leu^{26}-$\psi[CH_2NHCOCH_3]GRP_{19-26}$ peptides, **13–20**, were prepared by coupling Boc-leucinal directly to BHA resin, followed by reduction with $NaBH_3CN$.[27] We initially studied the stability of the Leu-$\psi(CH_2NH)$ bond that is formed between either the MBHA or the BHA resins and observed that the bond between the secondary amine and the BHA resin is more stable to repeated TFA treatment than with the MBHA resin. That is, there was loss of growing peptide from both resins, but more so from the MBHA resin. However, in order to obtain efficient HF

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Table II. Inhibition of Bombesin-Stimulated Amylase Release

peptide[a]	basal[b]	bombesin 0.2 nM	peptide only		peptide + BN (0.2 nM)	
			50 nM	5 μM	50 nM	5 μM
C	2.8 ± 0.3	17.4 ± 4.2	3.0 ± 0.6	2.7 ± 0.2	11.6 ± 1.5	6.2 ± 0.8
H	5.3 ± 0.5	17.4 ± 0.9	5.1 ± 0.5	6.0 ± 1.2	6.3 ± 0.8	6.3 ± 0.4
1	5.5 ± 0.5	18.1 ± 2.0	6.9 ± 1.4	22.3 ± 1.6	19.6 ± 2.5	23.8 ± 3.0
2	3.6 ± 0.9	15.6 ± 3.0	3.5 ± 0.6	6.0 ± 1.4	15.7 ± 1.9	14.1 ± 1.3
3	3.7 ± 0.1	10.4 ± 0.5	ND[c]	13.3 ± 0.1	ND	11.6 ± 2.4
4	3.7 ± 0.1	10.4 ± 0.5	ND	10.3 ± 0.3	ND	13.0 ± 0.4
5	3.5 ± 0.2	9.4 ± 0.3	ND	9.2 ± 0.9	ND	13.1 ± 0.5
6	3.5 ± 0.2	9.4 ± 0.3	ND	9.0 ± 1.2	ND	9.9 ± 0.9
7	3.5 ± 0.2	9.4 ± 0.3	ND	9.1 ± 0.6	ND	12.9 ± 0.5
8	5.1 ± 0.4	19.3 ± 2.2	7.6 ± 1.9	6.5 ± 0.9	22.7 ± 2.7	24.5 ± 6.1
9	4.9 ± 0.3	17.6 ± 1.7	5.4 ± 1.2	5.3 ± 0.5	20.8 ± 1.1	18.1 ± 4.1
10	5.3 ± 0.1	18.3 ± 4.5	5.3 ± 0.3	5.7 ± 0.4	19.6 ± 2.1	13.1 ± 2.2
11	4.8 ± 0.5	17.8 ± 1.2	4.7 ± 1.2	4.7 ± 0.1	17.9 ± 1.6	16.1 ± 1.1
12	6.4 ± 1.7	21.2 ± 2.6	7.2 ± 1.2	12.2 ± 1.5	16.0 ± 5.3	14.3 ± 1.4
13	3.1 ± 0.3	15.9 ± 0.3	ND	3.6 ± 0.7	12.2 ± 0.5	4.0 ± 0.3
14	3.0 ± 0.5	15.7 ± 0.4	ND	3.0 ± 0.5	9.0 ± 0.6	3.4 ± 0.5
15	3.6 ± 0.4	14.4 ± 1.4	ND	3.6 ± 0.4	14.5 ± 0.5	8.2 ± 2.3
16	2.9 ± 0.5	20.2 ± 1.0	ND	3.6 ± 0.8	16.7 ± 2.1	3.7 ± 0.9
17	3.0 ± 0.2	16.5 ± 0.9	ND	ND	14.8 ± 1.1	4.3 ± 0.8
18	3.3 ± 0.3	14.6 ± 1.1	ND	ND	10.3 ± 0.8	3.6 ± 0.6
19	3.2 ± 0.3	16.0 ± 1.8	ND	ND	15.8 ± 0.5	12.5 ± 0.8
20	3.2 ± 0.2	20.3 ± 1.1	ND	3.1 ± 0.2	16.2 ± 1.3	4.8 ± 0.2

[a] C, [Leu^{14},$\psi^{13,14}$]BN; H, *N*-pivaloyl-GRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide. [b] Values are expressed as percentage of amylase released (mean ± SD). All measurements were done in triplicate. [c] ND = not determined.

cleavage of the desired peptide from the resin, it was necessary to acetylate the $\psi(CH_2NH)$ resin-bound secondary amine. This acetylation was carried out, via acetic anhydride/pyridine, at the end of the peptide synthesis and just prior to HF cleavage. If the acetylation was performed earlier in the synthesis it was found that the resulting peptide–$\psi(CH_2NCOCH_3)$–resin bond was even more labile to repeated TFA treatments and resulted in greater loss of growing peptide from the resin. These studies suggest that peptides linked to either BHA or MBHA resins via a $\psi(CH_2NH)$ or $\psi(CH_2NCOCH_3)$ bond are much more labile to repeated TFA treatments than peptides linked via the usual amide linkage.

In the preparation of peptides **13–18** a common resin intermediate, [Boc-Trp(For)^{21},Leu^{26}-$\psi(CH_2NH)$]GRP$_{21-26}$-BHA, was used in all of their syntheses. After further elaborations, pertinent deblockings and acetylations the resin-bound peptides were cleaved from the resin by the low/high HF procedure, which reportedly removes the formyl blocking group.[33] In each case we observed, upon HPLC purification, incomplete removal of the formyl group from Trp. In the case of peptides **13** and **14** we isolated their corresponding formyl byproducts, **19** and **20**. The CID mass spectrum of peptide **20** showed an $(M + H)^+$ ion of m/z 1021.1 and identification of the formyl group on Trp^{21} was made by observation of an a_3 ion at 471.0 and a b_3 ion at 498.9. In comparison, the parent peptide **14** showed the $(M + H)^+$ ion at m/z 993.1, the a_3 ion at 442.9, and the b_3 ion at 470.9, all of which are 28 amu lower. The FABMS of peptide **19** also showed an $(M + H)^+$ ion that was 28 amu higher than its parent, peptide **13**, and therefore we also place the formyl group on the Trp^{21} residue.

Biological Results. All of the peptides were tested using a dispersed acini system from rat pancreas (Table II). The basal value represents that amount of amylase released from the acini incubating under identical conditions, but without secretagogue added, and therefore is the control value.[34] The percent amylase released from these acini was measured in order to estimate agonistic and antagonistic activity of each peptide, in the absence or presence of BN (0.2 nM), respectively. The concentration of BN used for the present study (0.2 nM) is close to the optimal concentration (~1 nM) and was chosen so that our results can be compared to those in the literature.[17,18,35] Inhibition of BN-stimulated amylase release would be a measure of antagonist action. The dispersed acini preparation from guinea pig and rat has been widely and successfully used for uncovering BN/GRP antagonists, as was reviewed by Jensen and Coy.[14] Those peptides found to be antagonists in the amylase release assay were further tested for their inhibition of BN-stimulated [^{3}H]-thymidine incorporation by serum-starved Swiss 3T3 fibroblasts. These results were compared with those of two known antagonists, [Leu^{14},$\psi^{13,14}$]BN[16] (Dr. David Coy, Tulane University Medical Center; peptide **C**) and *N*-pivaloyl-GRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide[7] (Dr. David Heimbrook, Merck Sharp & Dohme Labs; peptide **H**).

The data shown in Table II supports the following. All five peptides containing the $\psi(CH_2NCHO)$ unit (**3–7**) still retained agonist activity (at 5 μM) but showed no antagonist activity. Peptides **8–11**, which all contain the $\psi(CH_2NH)$ bond between Leu^{26} and Met^{27}, were neither agonists nor antagonists, while **12** had some agonist activity (only at 5 μM). Peptides **13–20**, which are devoid of Met^{27} and terminate with the $\psi(CH_2NHCOCH_3)$ group, were all shown to be antagonists. Furthermore, those that were tested, **13–16** and **20**, had no agonist action.

A dose–response study of the ability of peptides **13–20** to inhibit amylase release is shown in Figure 2. In this graph, all results are normalized to BN-stimulated amylase release alone (taken as 100%) because of individual variations of different dispersed acini preparations, which is an acceptable practice.[14,35] At a concentration of 5 μM,

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(34) Lee, P. C.; Jensen, R. T.; Gardner, J. D. Bombesin Induced Desensitization in Dispersed Acini from Guinea Pig Pancreas. *Am. J. Physiol.* 1980, *238*, G213–G218.

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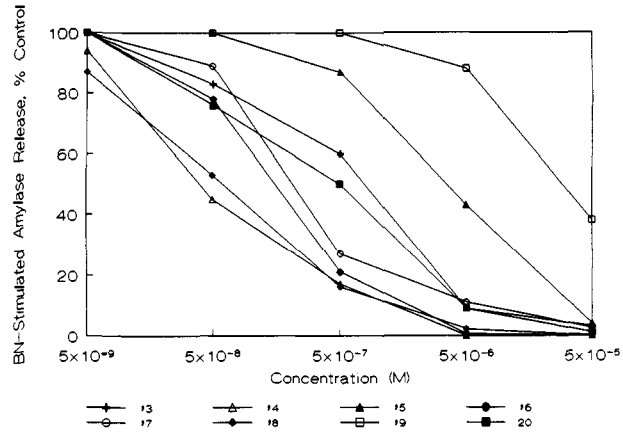

Figure 2. Dose–response study of peptides **13–20** in inhibiting amylase release from dispersed rat pancreatic acini. Amylase release is expressed as the percentage of the stimulated release caused by 0.2 nM BN with no analogue present. Each point represents the mean of three separate experiments, and the standard deviations were less than 10%.

peptides **14**, **16**, and **18** completely inhibited BN-stimulated amylase release. Clearly, peptides **14** and **18** are the most active, having IC_{50}s of 46 and 55 nM.

A dose–response study measuring the ability of peptides **13–20** to inhibit BN (10 nM) stimulated [^{3}H]thymidine incorporation into serum-starved 3T3 cells (control, 5488 CPM) is shown in Figure 3. For comparison purposes the results of the two known antagonists, **C** and **H**, are also included in this figure. All of the peptides showed dose-dependent antagonist activity. Again, compound **14** was the most potent antagonist.

Dose–response curves (expressed as percent inhibition of proliferation) and corresponding linear regression analysis lines for the two most potent antagonists of [^{3}H]-thymidine incorporation into serum-starved 3T3 cells, peptides **14** and **18**, as well as the two known peptide antagonists, **C** and **H**, are graphed in Figure 4. The IC_{50} values (denoted by the arrow) determined from these graphs for **14**, **18**, **C**, and **H** are 2.7, 32.5, 43.2, and 31.2 nM, respectively.

The IC_{50} values of peptides **13–20** and the two reference antagonists, **C** and **H**, for BN-stimulated [^{3}H]thymidine uptake in serum-starved 3T3 cells are summarized in Figure 5.

The specificity of peptide **14**, the most potent antagonist, for the BN/GRP receptor is shown in Figure 6. Using the dispersed acini system, **14** did not alter the stimulation of amylase release by CCK-8 (cholecystokinin C-terminal octapeptide), carbachol, A23187 (Calcimycin), substance P, or VIP (vasoactive intestinal polypeptide). However, **14** inhibited the stimulated amylase release by BN and NMC. This indicates that peptide **14** is a potent and specific BN/GRP receptor antagonist.

Conclusions

We have evaluated 20 GRP C-terminal analogues for their antagonist activity in two biological assays. On the basis of these assays we suggest that a des-Met^{27},Leu^{26}-ψ[$CH_2NHCOCH_3$]GRP C-terminal octapeptide imparts antagonist activity. This is in agreement with several studies,[13,14,17] the results of which conclude that the presence of the C-terminal Met is imperative for imparting agonist (biological) activity, but is not needed for affinity to the receptor. In fact, other amino acids can replace Met at the C-terminus and varying degrees of agonist

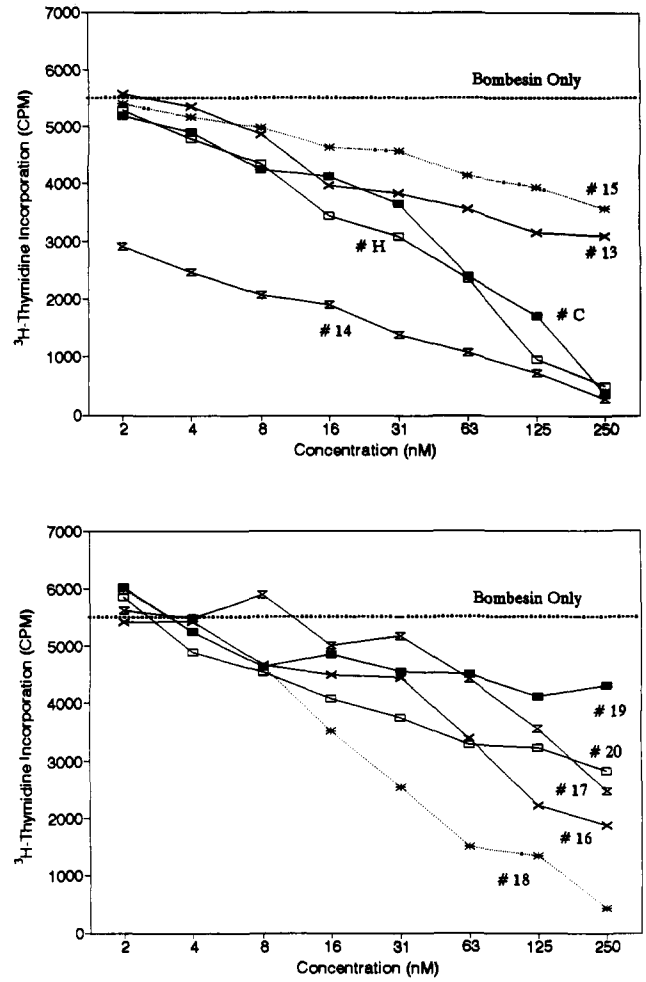

Figure 3. Dose–response study of peptides **13–20** and two known peptide antagonists, **C** (Leu^{14},$\psi^{13,14}$]BN)[16] and **H** (*N*-pivaloyl-GRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide)[7] in inhibiting BN (10 nM) stimulated [^{3}H]thymidine incorporation in serum-starved 3T3 cells (control, 5488 CPM). Each point represents the mean of three separate experiments, and the standard deviations, in CPM, were less than 10%.

activity is retained.[13,14,36] The wide variety of chemical changes made in the terminal Leu moiety of des-Met BN/GRP analogues[7,13,14] (e.g., esters, amides, and carbonyl reduced derivatives), with retention of varying degrees of antagonist activity, suggests that antagonist activity and thus binding to the BN/GRP receptor is highly dependent on the group attached to the BN^{12}/GRP^{25} terminal His. Heimbrook et al.[7] proposes, for receptor binding affinity, that the optimal length of the group attached to histidine is one which "may mimic the α-carbon backbone of the Leu^{26}-Met^{27} portion of native GRP".

In the series **13–20** the N-terminus should not be acylated, replacement of Asn^{19} with D-Phe^{19} gives a potent antagonist while Gln^{19} does not. The combination of D-Phe^{19},His^{20} has a slightly better antagonist effect than does D-Phe^{19},Gln^{20}. In a similar comparison of the antagonist activity of [D-Phe^2,Leu^{10},$\psi^{9,10}$]NMC_{2-9} (His) vs [D-Phe^6,Leu^{13},$\psi^{13,14}$]BN_{6-14} (Gln) analogues, Coy et al.[37] observed that Gln was favored by a factor of 5.

In conclusion, the novel C-terminal ψ[$CH_2NHCOCH_3$] bond promises to be a useful peptide backbone modification for imparting antagonism in GRP/BN analogues.

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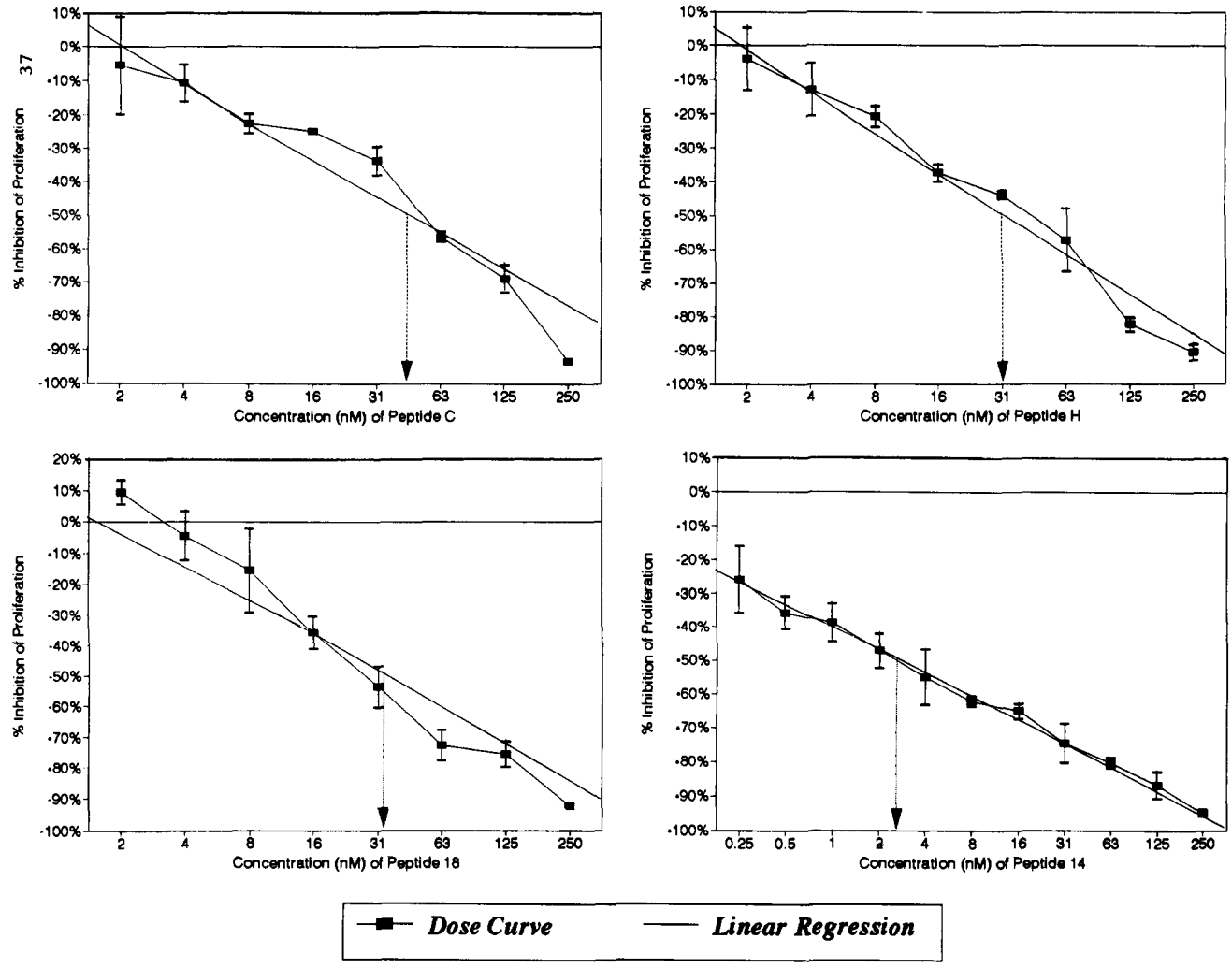

Figure 4. Dose–response curves (inhibition of BN (10 nM) stimulated [^{3}H]thymidine incorporation in serum-starved 3T3 cells expressed as percent inhibition of proliferation) and corresponding linear regression analysis lines of the two most potent antagonists, peptides 14 and 18, compared to two known peptide antagonists, C (Leu^{14},$\psi^{13,14}$]BN)[16] and H (*N*-pivaloyl-GRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide).[7] The IC_{50} values (denoted by the arrow) for 14, 18, C, and H are 2.7, 32.5, 43.2, and 31.2 nM, respectively.

These compounds have the potential of inhibiting the growth of SCLC.

Experimental Section

The chemicals and solvents were purchased from the following sources: Boc-amino acids were of the L configuration, unless stated otherwise (Bachem, Sigma, and Vega); TFA and diisopropylethylamine (DIEA) (Fisher Biotech); DCM, HPLC-grade acetonitrile (CH_3CN), and triethylamine (TEA) (Fisher); anisole, dimethyl sulfide (DMS), *p*-cresol, *p*-thiocresol, 1,3-diisopropylcarbodiimide (DIC), *O*,*N*-dimethylhydroxylamine hydrochloride, HOBt, Amberlite IR-120 resin, and sodium cyanoborohydride (Aldrich); MBHA and BHA resins (1% cross-linked, 200–400 mesh), and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, Bachem); HF (Matheson); cobaltic fluoride (CoF_3) and lithium aluminum hydride (LAH) (Alfa); DMF (Aldrich, Baxter or B & J); dicyclohexylcarbodiimide (DCC) (Chemalog); phenol and acetic anhydride (Mallinckrodt); mixed bed resin (20–50 mesh, Bio-Rad). Preparative (on glass, 1 mm thick) and analytical (on aluminum) silica gel TLC plates were obtained from Analtech. The following were purified by distillation: DCM from anhydrous Na_2CO_3; TEA, DIEA, and pyridine distilled from ninhydrin; acetic anhydride in vacuo. All the peptides were synthesized in a Milligen 504 synthesizer (manual). The apparatus for conducting the liquid HF cleavages was constructed as previously described.[38] The HF was dried prior to use by distilling it into the first vessel which contained CoF_3 and then by distilling from there into the reaction vessel. Analytical and semipreparative HPLC separations were performed on a Waters Associates system (consisting of a U6K injector, M45 and 510 pumps, an automated gradient controller, and a Model 484 tunable absorbance detector connected to a Hitachi Model D-2000 Chromato-Integrator), using Vydac C_{18}, 5-μm particle size, 300-Å pore size columns, 0.46 × 15 cm (UV detection at 214 nm) and 1.0 × 25 cm (UV detection at 280 nm), respectively. The gradients used were linear mixtures of solvent A (0.05% TFA in H_2O) and solvent B (0.05% TFA in CH_3CN or 60% CH_3CN/H_2O). The columns were protected with a Vydac guard cartridge containing the same packing, but of 10-μm particle size. The peptide samples, after separation from HPLC, were lyophilized on a Virtis freeze dryer. Amino acid analyses were performed, after hydrolysis of the peptides, on a Beckman Model 6300 analyzer. The hydrolyses were accomplished in one of three different systems (6N HCl containing 5% phenol, methanesulfonic acid, or 6 N HCl containing 5% thioglycolic acid) for 24 h, at 110 °C, in vacuo. The reagent containing 5% thioglycolic acid was used for compounds 13–20 in order to obtain better results for Trp.[39] Fast atom bombardment mass spectrometry (FABMS) spectra were determined on an Extrel Model ELQ 400 spectrometer, using glycerol as the liquid matrix. The CID-MS were recorded at the Massachusetts Institute of Technology (MIT) Mass Spectrometry Facility.

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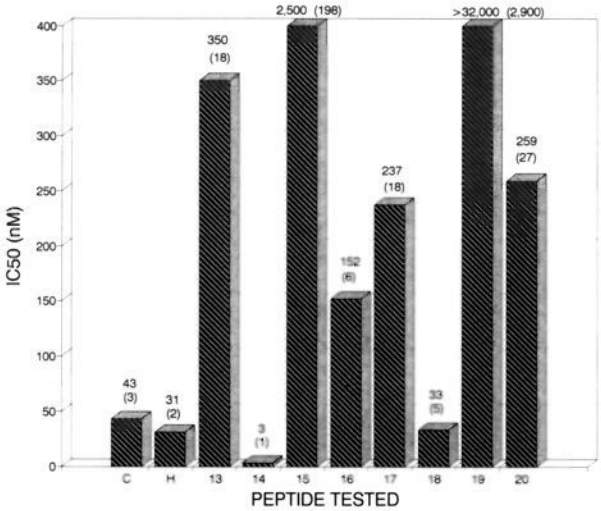

Figure 5. Comparison of the IC_{50} values (from linear regression analysis of the data in Figures 3 and 4) of peptides **13–20** and two known peptide antagonists, **C** (Leu^{14},$\psi^{13,14}$]BN)[16] and **H** (*N*-pivaloylGRP$_{20-25}$-(*R*)-2-methyl-4-nonylamide)[7] for stimulated [^{3}H]-thymidine uptake in serum-starved 3T3 cells. The standard deviation of each IC_{50} value is given in parentheses.

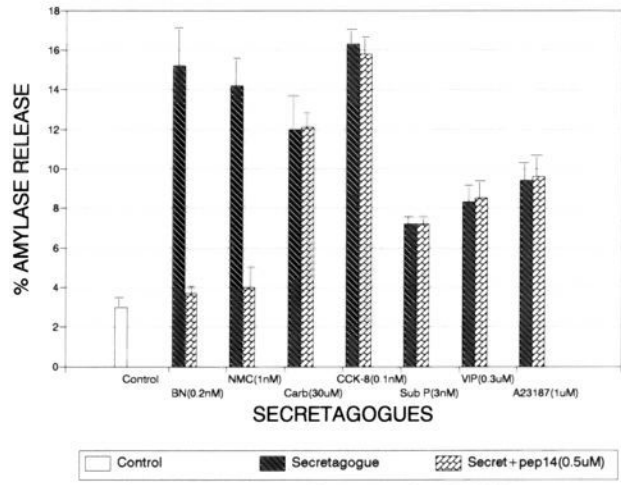

Figure 6. Specificity of peptide **14** for the BN/GRP receptor. The ability of various secretagogues to stimulate amylase release from dispersed rat pancreatic acini was not inhibited by coadministration of peptide **14**, at 0.5 μM, except in the cases of BN and NMC.

Boc-leucinal. This aldehyde was prepared by a slight modification of the method of Fehrentz and Castro.[28] Boc-Leu hydrate (1.75 g, 7.0 mmol) was converted to anhydrous Boc-Leu by repeated evaporations from 10-mL portions of toluene. The resulting oil was dried over P_2O_5 in a vacuum desiccator and converted to Boc-Leu-*N*-methoxy-*N*-methylamide in a 97% yield.[28] Reduction of this amide with $LiAlH_4$ gave the desired Boc-leucinal in a 57% yield; the ^{1}H NMR spectrum agreed with that previously reported.[28] The procedure of Fehrentz and Castro[28] leads to Boc-protected aldehydes which show high optical purity. We did not determine the enantiopurity of Boc-leucinal, always prepared it fresh, and used it immediately in the preparation of the $\psi(CH_2NH)$ peptide bond.

Solid-Phase Synthesis. Boc-L-amino acids were used for all of the syntheses, and protection for the side-chain functionalities was Arg(Tos), His(Bom), Trp(For), and where appropriate Boc-Trp, Boc-D-Trp(For), Boc-D-Phe, Boc-L-*N*-Me-Phe, Boc-D-*N*-Me-Phe, or Boc-D-Arg(Tos) was used. Generally, the synthesis was started by loading MBHA (0.68 mequiv/g, peptides **1–12**) or BHA (0.89 mequiv/g, peptides **13–20**) resin into the Milligen manual synthesizer followed by washing the resin with DCM, 5% DIEA/DCM, and DCM. In the preparation of peptides **1–12** the first amino acid was coupled as its preformed symmetrical anhydride (PSA) in a 3-fold excess in DCM.[40] The PSA mixture was placed into the barrel of a 10-mL syringe fitted with a 0.45-μm syringe filter and filtered (DCU removed) directly into the Milligen flask. The coupling was allowed to proceed for 2 h, and the growing peptide–resin was then washed with DCM, 5% DIEA/DCM, and DCM. To evaluate the extent of coupling a 1–3-mg portion of dried resin was removed and subjected to quantitative ninhydrin analysis.[22] If the test was positive, a second coupling, in a 3-fold excess, was performed but with the amino acid as its preformed HOBt ester.[40] The HOBt esters were preformed in DMF/DCM (2/1) and filtered, as described above for the PSA, directly into the Milligen flask. Coupling in DMF was allowed to proceed for 2 h. After appropriate washings the Boc group was cleaved with 50% TFA/DCM. Subsequent residues were coupled as above, except if they were Arg(Tos), Asn, Gln, Gly, or His(Bom). In those cases they were single or double coupled as their preformed HOBt esters. After the last residue was added, the N-terminal Boc group was cleaved as usual and the resin dried prior to HF cleavage. In peptides **3–12** Trp was incorporated with its side chain unprotected, thus Boc group cleavages following Trp introduction used 50% TFA/DCM containing 3% anisole and 0.2% dithiothreitol to inhibit alkylation of the indole ring. In the case of peptides **3–12** the $\psi^{26,27}$ bond was formed by reacting Bos-leucinal (2.5 equiv) with the free amino group of Met-MBHA, according to the method of Sasaki and Coy.[27] After the introduction of the $\psi^{26,27}$ bond, the incorporation of Gly into the peptides was via Boc-Gly-ONp (5 equiv) in DMF.[27] The acetyl group in peptide **11** was introduced by reaction of Trp-peptide-MBHA with Ac-OBt in DMF. Peptides **1–12** were cleaved from the resin with 10% anisole/HF at −10 to 0 °C for 1 h.

In peptides **13–20** the Leu^{26}–$\psi(CH_2NH)$ bond was formed by reacting BHA resin directly with Boc-leucinal (3.0 equiv) in DMF containing 1% AcOH followed by reduction with a solution of $NaBH_3CN$ in DMF containing 1% AcOH during 3 h.[27] The reaction was repeated with half the amount of Boc-leucinal (1.5 equiv) for 4 h. Capping of any free primary amine still available on the resin was accomplished by the addition of Z-Leu symmetrical anhydride (3.3 equiv) for 3 h; acetic anhydride was not used so as to prevent acylation of the $\psi(CH_2NH)$ group just formed. The peptides were elongated using the procedures outlined above, except that Trp(For) was used. In the preparation of peptides **14**, **15**, **18**, and **20**, the fully blocked peptide-resin was acetylated with a solution of acetic anhydride/pyridine/DMF (1:1:2) for 17 h. After appropriate washings the terminal Boc group was cleaved with 50% TFA/DCM. All of the TFA filtrates were collected and evaporated to an oil, which was solidified by the addition of ether. The resulting white solid, which is a partially deblocked peptide that was cleaved from the resin, and the remaining peptide-resin were combined and subjected to low/high HF cleavage,[41] as follows. The solids were placed in the HF apparatus[38] along with 0.25 mL each of *p*-cresol and *p*-thiocresol, 3.25 mL of DMS and then 1.25 mL of HF were added, and cleavage was carried out for 2 h at −10 to 0 °C. The HF and DMS were removed in vacuo, and a fresh portion (4.5 mL) of HF was distilled into the vessel and cleavage carried out for 1 h at −10 to 0 °C. After removal of the HF in vacuo, the solids were extracted with EtOAc to remove *p*-cresol and *p*-thiocresol. The remaining solids were extracted with 30% AcOH, and the aqueous layer was lyophilized. In the preparation of peptides **13**, **16**, **17**, and **19**, the fully blocked peptide-resin was treated first with 50% TFA/DCM to cleave the N-terminal Boc group, neutralized with 5% DIEA/DCM, and then acetylated as above with acetic anhydride/pyridine/DMF (1:1:2). After washing with DMF, the dried acetylated-peptide-resin was fully deblocked and cleaved from the resin by the above-described low/high HF method.[41]

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Table III. Characterization Data of BN/GRP Peptides

peptide no.	calcd Mr	obsd FABMS	amino acid analysis								
			Ala	Asx	Gly	His	Leu	Phe	Trp	Val	other
1	1198.6	1200.0	1.00	0.96	2.00	0.77	1.97	1.00	ND		
2	1354.7	1356.0	1.00	1.08	2.00	1.02	2.06	1.03	1.53		
3	1166.6	1167.8	1.05		2.05	1.98		1.03	ND	1.06	
4	1109.6	1110.5	1.03		1.03	1.92		1.00	ND	1.00	
5	1133.5	1135.5	1.00	1.07	2.07	1.85			0.96	0.96	
6	1123.5	1124.7	1.04		1.00	1.88			ND	1.00	*N*-Me-Phe 0.55
7	1123.5	1123.0	1.03		1.01	1.82			ND	1.00	*N*-Me-Phe 0.66
8	968.5	969.0	1.00	0.85	1.85	0.82			1.01	1.00	
9	797.0	798.0	0.93		0.94	1.07			1.15	1.00	
10	944.0	945.0	1.00		0.97	0.74		1.15	0.98	1.00	
11	839.0	840.0	0.94		0.92	1.31			1.22	1.00	
12	958.0	959.0	0.83		0.77	1.23			1.30	1.00	*N*-Me-Phe 1.11
13	1015.5	1016.0	0.98		0.92	1.56			1.12	1.00	Gly 1.09
14	992.5	993.1	1.00		1.00	2.10		1.00	1.03	1.03	
15	973.5	975.0	1.00		0.96	1.98			1.48	1.01	Gly 1.13
16	1034.5	1037.0	1.08		1.00	1.86		1.10	1.14	1.03	
17	1025.5	1027.0	1.00		0.95	0.88		1.05	1.12	0.98	Gly 1.04
18	983.5	985.0	1.00		1.00	0.92		1.08	1.12	1.02	Gly 1.10
19	1043.5	1045.0	1.00		0.96	1.98			1.49	1.01	Gly 1.13
20	1020.5	1021.1	1.00		0.97	1.90		1.10	1.18	0.98	

Each of the peptides were purified by reversed-phase HPLC, and their purity was at least 95%. Amino acid analysis and FABMS, which was especially needed in the case of the pseudopeptides, were used to confirm the structure of each peptide and the results are shown in Table III.

Biological Studies. Amylase Release. Adolescent Sprague-Dawley rats of both sexes (75–100 g) were sacrificed by decapitation. The pancreata were immediately dissected and trimmed of fat and mesentery. Dispersed pancreatic acini were prepared from pancreata by controlled collagenase digestion according to a previous method.[34] Briefly, the procedure involved multiple injections of a collagenase solution (300 units/20 mL) into the interstitium of the pancreatic tissue, followed in succession by incubation and mechanical dispersion. The dispersed acinar preparation was passed through 4% BSA (W/V) in an incubation buffer three times to remove collagenase. The final acinar preparations were used for the studies of amylase release using different concentrations and combinations of secretagogues and various test peptides, as specified. The standard incubation buffer contained 25 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM sodium phosphate monobasic, 5 mM Na pyruvate, 5 mM fumarate, 5 mM glutamate, 11.5 mM glucose, 0.5 mM $CaCl_2$, 2 mM glutamine, 1% albumin, 0.01% soy bean trypsin inhibitor, 1% essential amino acid mixture, and 1 mg/mL bacitracin. In all incubations, the gas phase was 100% oxygen, and all solutions were equilibrated with 100% oxygen.

The release of amylase into the extracellular medium in the presence and absence of secretagogue and/or peptide was measured as reported previously.[34] Amylase secretion was calculated as the percent of amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during a 30-min incubation at 37 °C under an atmosphere of 100% oxygen. Amylase activity was measured using the Phadebas amylase test from Pharmacia Diagnostics.

Proliferation of 3T3 Cells in Response to Bombesin and Antagonists. Day 1. Normal 3T3 cells were plated at a concentration of 2×10^4 cells/well in flat-bottom microtiter plates in Dulbecco's modified eagle media (DMEM) plus 10% serum and incubated at 37 °C in 5% CO_2.

Day 2. Wells were checked to ensure that the 3T3 cells had reached confluency. Media was poured off and 0.2 mL of serum-free medium was added to each well. Plates were incubated as above.

Day 4. (1) BN, experimental peptides, and [^{3}H]thymidine were diluted to appropriate concentrations in serum-free medium. (2) The medium was removed from the experimental wells, and the following components were added: (a) 0.05 mL of serum-free medium, (b) 0.05 mL of experimental peptide (dilutions), (c) 0.05 mL of BN (10 nM final concentration), (d) 0.05 mL of [^{3}H]-thymidine (1 mCi/50 mL). Each experimental peptide dilution was evaluated in three replicate wells. (3) The medium was removed from the control wells, and the following components were added: (a) no serum/no BN (0.15 mL of serum-free medium + 0.05 mL of [^{3}H]thymidine), (b) + serum/no BN (0.13 mL of serum-free medium + 0.02 mL of serum + 0.05 mL of [^{3}H]-thymidine), (c) no serum/+ BN (0.1 mL of serum-free medium + 0.05 mL of BN + 0.05 mL of [^{3}H]thymidine), (d) + serum/+ BN (0.08 mL of serum-free medium + 0.02 mL of serum + 0.05 mL of BN + 0.05 mL of [^{3}H]thymidine). Six replicate wells were evaluated for each control condition.

Day 6. The media from the wells were removed, and 0.01 mL of trypsin–EDTA (0.05 % trypsin, 0.53 mM EDTA) was added to each well. The plates were incubated for 10 min at 37 °C and the contents of the wells harvested onto filters, dried, and counted for radioactive incorporation.

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